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SURVEY AND SUMMARY

Chromatin remodeling finds its place in the DNA double-strand break response

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ABSTRACT

The accurate repair of chromosomal double-strand breaks (DSBs) arising from exposure to exogenous agents, such as ionizing radiation (IR) and radiomimetic drugs is crucial in maintaining genomic integrity, cellular viability and the prevention of tumorigenesis. Eukaryotic cells have evolved efficient mechanisms that sense and respond to DSBs. The DNA DSB response is facilitated by hierarchical signaling networks that orchestrate chromatin structural changes, cell-cycle checkpoints and multiple enzymatic activities to repair the broken DNA ends. Sensors and transducers signal to numerous downstream cellular effectors which function primarily by substrate posttranslational modifications including phosphorylation, acetylation, methylation and ubiquitylation. In particular, the past several years have provided important insight into the role of chromatin remodeling and histones-specific modifications to control DNA damage detection, signaling and repair. This review summarizes recently identified factors that influence this complex process and the repair of DNA DSBs in eukaryotic cells.

INTRODUCTION

Cellular DNA can be damaged by physiological processes and environmental agents, resulting in a variety of lesions, including DNA base modifications, crosslinks and single- and double-strand breaks (SSBs and DSBs). Ionizing radiation (IR) as well as radiomimetic drugs create reactive forms of oxygen, which in turn attack DNA and lead to strand breakage in all phases of the cell cycle (1,2). DSBs produced by IR can be complex often resulting in end modifications, attachment of bulky adducts or some loss of DNA in the vicinity of the damage. At low doses of IR, single-strand nicks predominate although nearby nicks on

two complementary strands of the DNA double helix can be converted into DSBs. The exact mechanism of IR-induced DNA DSB formation is not yet clear; however, both free radicals and direct ionizations are involved. The number of DNA DSBs per mammalian cell detected immediately after 1 Gy (100 rads) exposure is approximately 40, whereas the same IR dose results in approximately 1000 SSBs and 1000 damaged bases. DNA DSBs are considered the critical primary lesions in the formation of chromosomal rearrangements associated with disease and tumorigenesis.

DSBs are also generated in a programmed manner to initiate recombination between homologous chromosomes during meiosis (3) and as intermediates during V(D)J recombination and immunoglobulin (Ig) class-switch recombination (4,5). While programmed rearrangements are initiated by specific enzymes that generate DNA DSBs in the target locus, e.g. RAG1 and RAG2 in V(D)J recombination, the recombination intermediates seem to be resolved by the same pathways used to repair IR-induced DNA DSBs (4). DNA DSBs can arise also at sites of DNA replication when replication forks encounter DNA SSBs or other lesions, either interstitial or at the ends of chromosomes due to defective telomere metabolism (6,7).

IR-induced DSBs are repaired by either homologous recombination (HR) or non-homologous end joining (NHEJ) (8–13). NHEJ requires little or no DNA sequence homology at the damaged ends, and the proteins implicated in NHEJ generally act to maintain physical proximity of the two broken ends on one DNA duplex during minimal processing followed by ligation. In contrast, HR requires a sister chromatid, homolog or homologous sequence on a heterolog to be used as a template for repair. This pathway requires more significant chromatin remodeling and access to DNA bases to facilitate DNA unwinding, strand invasion and DNA replication-based repair mechanisms. Initial insights into HR came from the characterization of the yeast *RAD52* epistasis group of genes, first isolated from yeast mutants hypersensitive to X-rays. The mammalian homologs have been identified

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and their genetic and biochemical roles in DNA repair mechanisms are well described (14–17).

It is now appreciated that chromatin structure has an integral role in DNA DSB repair (18) and that the chromatin response, in general, may precede DNA end resection (19). DNA DSBs lead to redistribution of DNA damage response (DDR) proteins into dynamic, higher order multi-protein assemblies on chromatin surrounding the break. Adaptor proteins are crucial to build these assemblies in a spatial and temporal manner, in part, by hierarchical phosphorylations that are selectively recognized by downstream DDR proteins. The inability to respond properly to DNA DSBs or to repair them has the potential to lead to cell death, genomic instability or malignant transformation. There are a number of human disorders characterized by defects in proteins that function in DSB repair or that are characterized by an altered ability to interact with chromatin to modulate the repair process. The study of these disorders has provided important insight into the cellular response to DSBs.

INITIAL DETECTION OF DNA DSBs

The DDR operates through the hierarchical action of sensors, transducers and effectors that orchestrate the repair of DNA DSBs. Detection of a lesion is the first essential step in the cellular response to DSBs. The Mre11 complex has been implicated as having an early role in the detection of DSBs (20,21). Recruitment of the Mre11 complex and activation of ataxia-telangiectasia mutated (ATM) protein are both rapid events and interdependent in both recognition and signaling of DNA DSBs (22). Additional proteins assemble within the entire regions of modified chromatin up to 2 Mb from the DSB (23) suggesting the potential significance of modifications in chromatin structure also acting as DSB sensors.

DNA DSBs lead to redistribution of proteins into dynamic, higher order multi-protein assemblies on chromatin, often detected by IR-induced immunofluorescent foci (IRIF). Proteins can be categorized on the basis of spatial redistribution: (i) proteins associated with DSB-flanking chromatin (Mre11, ATM, Nbs1, Rad50, DNA-PKcs, Ku70/80, XRCC4, Mdc1, 53BP1 and BRCA1); (ii) proteins associated with single-stranded DNA (ssDNA) micro-compartments [*ATM- and Rad3-related* protein (ATR), ATRIP, RPA, Rad9, Rad17, Rad51, Rad52, BRCA2, FANCD2, Nbs1, Mre11, Rad50, BRCA1]; and (iii) proteins that do not form cytologically detectable IRIF (Smc1, Smc3, Chk1, Chk2, p53, Cdc25A). Chromatin marked by the phosphorylated form of H2AX (γ -H2AX) becomes occupied by ATM, Mdc1 and 53BP1, whereas proteins involved in repair (Rad51, Rad52, BRCA2 and FANCD2, ATR and RPA) and the DNA clamp proteins, such as Rad17 and Rad9 accumulate in chromatin micro-compartments delineated by ssDNA (24). BRCA1 and the MRN complex interact with both of these compartments. Smc1 and Smc3 associate with chromatin as preassembled complexes, but become locally modified after DNA damage. p53 and

Cdc25A are involved in cell-cycle checkpoint activation but do not accumulate at DNA DSB sites (24).

Mre11 complex

A significant amount of data suggests that Mre11, Rad50 and Xrs2/Nbs1 (Xrs2 in *Saccharomyces cerevisiae*; Nbs1 in mammalian cells) comprise a nuclease complex (also termed the Mre11- or MRN complex) with multiple roles in signaling and repair in meiotic and mitotic DSB response networks, as well as in telomere maintenance (14). The Mre11 complex is involved in early steps of DSB end processing prior to repair by multiple pathways and may maintain sister chromatids or broken ends in close proximity. These proteins bind DNA quickly after DSB induction, and influence the rate of 5' to 3' resection along with the yeast Sae2/Com1 (25). While all evidence indicates that a heteroduplex is required for the initial processing of broken DNA ends, the nature of the processing is unclear. The nuclease activity of Mre11 degrades DNA 3'–5', leading one group to hypothesize that the complex recruits an unidentified nuclease of the correct polarity or that the helicase activity of the complex provides a substrate for the endonuclease activity of Mre11 (26–29). Exo1 can partially compensate for loss of Mre11 activity in mitotic cells but not in meiotic cells suggesting that Mre11 acts differently in the context of Spo11-mediated DSBs (27,30). Initial studies showed a requirement for the Mre11 complex after IR exposure, which produces multiple types of DNA ends, raising the possibility that processing is limited to an initial 'cleaning' or removal of damaged ends rather than extensive nuclease activity. Interestingly, the proposed activity of Mre11 in meiosis is to assist in removal of Spo11 from DNA after cleavage [reviewed in (3)], analogous to removal of a bulky adduct from a DNA end in somatic cells. Recent studies have further revealed that CtIP (mammalian homolog of Sae2/Com, also termed RBBP8) confers resistance to DSB-inducing agents, physically and functionally interacts with the Mre11 complex and BRCA1, and both CtIP and Mre11 are required for efficient DNA-end resection, recruitment of the ssDNA binding protein RPA and HR in a cell-cycle-dependent manner (29,31,32). Similar results have been obtained with *Schizosaccharomyces pombe* Ctp1, a protein that shares conserved domains with CtIP (31).

Besides a role for initial processing of broken ends, the Mre11 complex has been postulated to tether broken DNA ends together to inhibit aberrant joining to heterologous chromosome sequences that would lead to genome rearrangements, such as translocations. The crystal structure of the Mre11 complex revealed strong similarities to the Smc proteins that tether replicated homologs together until anaphase signaling initiates chromosome separation. When complexed as a trimer, the N- and C-termini of Rad50 associate together separated by a long flexible arm with zinc hook at the tip (33–35). This structure along with initial genetic studies conducted with *MRE11S* and *RAD50S* yeast mutants led to the suggestion that heterotrimers associate at the zinc hooks during S phase to tether two chromatids together (26).

Furthermore, electron microscopic studies in mammalian cells suggest that multiple complexes associate to hold together the two ends of a broken DNA duplex (33,36). However, this may be an oversimplified model. Fluorescent tagging of individual DSBs in living mammalian cells has revealed that the positional stability of DSB ends is not affected by siRNA knockdown of H2AX, any member of the Mre11 complex or Smc1, but is significantly impaired by loss of Ku80 suggesting a key role for this protein in tethering ends in mitotic mammalian cells at least in some contexts (37). The relative stability of mammalian DSB ends contrasts with studies in *S. cerevisiae* in which ends are more mobile and coalesce into repair foci at a high frequency (38). Given that several genetic studies have demonstrated that DSBs stimulate interchromosomal HR and NHEJ repair mechanisms (39–41), these studies highlight our lack of understanding about what circumstances allow for the physical interactions necessary to facilitate interchromosomal repair of DNA damage.

Although null mutants of each of the three proteins of the Mre11 complex are embryonic lethal in mice, several hypomorphic strains have been engineered that are viable (42,43). As expected, these mice exhibit meiotic defects, as well as IR sensitivity and predisposition to cancer similar to the cognate human syndromes. Interestingly, the Rad50S-mutant mice display a distinct hematopoietic cell defect and bone marrow failure (43). The reason for the defect remains unclear, and possibilities include a specific role for Rad50 in tissue-specific stem cell proliferation or differentiation, or alternatively, a role in anti-apoptotic signaling. Since hematopoietic cells are particularly susceptible to apoptosis after DNA damage, partial loss of Rad50 function may be sufficient to demonstrate this phenotype. Taken together with elevated levels of apoptosis in other DDR mutants, such as mammalian Spo11^{-/-} spermatocytes and oocytes, ATM^{-/-} human neural cells and MEI304 *Drosophila* mutants, unrepaired DSBs in both meiotic and mitotic systems are sufficient to lead to apoptosis (44,45).

CHROMATIN MODIFICATION IN RESPONSE TO DNA DSBs

Chromatin creates a natural barrier against access to DNA during transcription, damage repair and recombination. There is an increasing body of evidence about the role of histone modifications in DDR (46–49) and DSB repair (50–52). Following DNA damage, chromatin structure is altered by (i) ATP-dependent chromatin remodeling, (ii) incorporation of histone variants into nucleosomes and (iii) covalent histone modifications (53,54). Among the different histone modifications, phosphorylation of all four histones as well as the variant H2AX plays a primary role in DDR by facilitating access of repair proteins to DNA breaks. There is also a strong correlation between defective DSB repair, genomic instability and telomere dysfunction, and further investigation into this area would determine whether telomere stability is based on the same paradigms (49,55,56).

Table 1. Histone modifications and associations in DSB response

Histone	Modification or association
H1	Phosphorylation by DNA-PK
H2A	Acetylation by TIP60
H2AX	Phosphorylation S139 by ATM Ubiquitylation S139 by RNF8
H3	Acetylation by TIP60 Methylation K79 by DOT1L Association of 53BP1 to H3K79me Association of RAG2 to H3K4me3
H4	Phosphorylation of S1 by ck2 Acetylation by NuA4/TIP60-TRAPP Acetylation K16 by hMOF Association of HP1 β to H4K20me3 Association of 53BP1 to H4K20me2

The histone modifications and associations related to the DDR are summarized in Table 1.

ATM and histone phosphorylation

One immediate target of the ATM kinase following DNA damage is the histone H2A-variant H2AX (57). Histone H2AX, the major isoform in yeast and a minor H2A species in mammals, is phosphorylated at the carboxy-terminal serine 139 in somatic cells in response to damage-induced DSBs (58), following Spo11-induced DSB formation (59,60), and following Rag-mediated cleavage (61). Phosphorylated H2AX (γ -H2AX) appears within minutes of damage over large adjacent chromatin regions extending tens of kilobase in yeast and up to 2 Mb in mammalian cells (23). Analysis of H2AX-deficient mice has demonstrated a role for the protein in a variety of responses to DSBs, including DNA repair, checkpoint signaling and Ig gene class switching (62–66). H2AX^{-/-} mice exhibit male-specific sterility, likely due to defects in chromatin remodeling during meiosis (65). γ -H2AX immunostaining of mouse spermatocytes is detected in leptotene and zygotene, and co-localizes with Rad51/Dmc1 foci. In contrast, staining is nearly absent at all times in SPO11^{-/-} mutants that do not form DSBs. Coating of large chromatin regions by γ -H2AX may act as a structural signal to recruit recombination and repair proteins, as well as the structural proteins involved in chromatid pairing and synaptonemal complex (SC) formation in pachytene. Although γ -H2AX is central to the DDR, mutation of the H2AX phosphorylation site confers only a moderate sensitivity to DNA damage in comparison to alterations of DNA damage checkpoint proteins (67,68). This suggests that γ -H2AX contributes to the process of DNA DSB repair; however, it is not essential since it is not required for the initial recognition of DNA breaks (67).

In addition to damage-induced H2AX phosphorylation, histone H4 phosphorylation at serine 1 by casein kinase 2 (ck2) occurs in response to DSBs, and serine 1 phosphorylation is reported to inhibit histone H4 acetylation by NuA4 acetyltransferase (69,70). These events may regulate restoration of chromatin structure following repair (70).

Interestingly, chromatin immunoprecipitation experiments have shown that H4 phosphorylation is also abundant near endonuclease-induced DSBs (69,70). There are several other chromatin modifying factors with chromodomains, e.g. isoforms of heterochromatin protein 1 (HP1), which have been linked with the repair of IR-induced DNA damage (49). Consistent with the observations that overexpression of HP1 β effects DNA DSB repair, Goodarzi and coworkers (71) demonstrated that heterochromatic DSBs are generally repaired more slowly than euchromatic DSBs, and ATM signaling is specifically required for DSB repair within heterochromatin. The isoforms of HP1 have potential ATM phosphorylation sites but it remains to be determined if these isoforms interact with ATM or other DDR elements.

Our studies suggested that ATM deficient A-T cells are more proficient in translating IR-induced DNA damage into chromosome damage possibly due to an underlying alteration in chromatin structure (1,5,72). In undamaged cells, ATM is held in an inactive form as a dimer or higher order multimer, with the kinase domain bound to a region surrounding serine 1981. Upon IR exposure ATM becomes rapidly autophosphorylated at serine 1981 causing dimer dissociation and initiating cellular ATM kinase activity (73). Recent studies have shown that DSB formation is followed by ATM-dependent chromatin relaxation. Following IR exposure, KRAB-associated protein (KAP-1, also known as TIF1 β , KRIP-1 or TRIM28) at the damage sites is immediately phosphorylated in an ATM-dependent manner on serine 824, and phosphorylated KAP-1 then spreads rapidly throughout the chromatin. Ablation of the KAP-1 phosphorylation site leads to loss of DSB-induced chromatin decondensation and renders the cells hypersensitive to DSB-inducing agents (74). KAP-1 also recruits HP1 proteins to form small HP1-containing heterochromatin domains that repress gene activity (75,76), and expression of mutant HP1 β results in abrogation of damage-induced H2AX phosphorylation (77). Further studies have shown that DNA damage influences the chromatin-associated movement of HP1 β , supporting the relationship between HP1 β and DDR (77). HP1 β co-localizes with trimethylated lysine 20 of histone H4 (H4K20me3) which is also a binding motif for 53BP1 (78). Thus, these studies strongly argue that cells deficient in ATM have defective chromatin relaxation, an essential initial step for the recognition and repair of DNA DSBs (74).

hMOF influences ATM function

hMOF, the human ortholog of the *Drosophila MOF* (males absent on the first), encodes a histone acetyltransferase (HAT) that interacts with ATM (79). Expression of a dominant negative hMOF mutant or RNAi-mediated hMOF knockdown blocked IR-induced increases in histone H4 acetylation at lysine 16 and resulted in decreased ATM autophosphorylation, ATM kinase activity, phosphorylation of downstream effectors of ATM and DNA repair, while increasing sensitivity to IR-induced cell killing. Ablation of MOF by gene targeting resulted in early embryonic lethality and cell death in mice (80).

Lethality correlated with the loss of H4K16 acetylation and could not be rescued by concomitant inactivation of ATM or p53 (80). In addition, decreased hMOF activity was associated with loss of the cell-cycle checkpoint response to DSBs. Taken together, these results suggest that hMOF functions upstream of ATM and its modification of histone H4 may contribute to DSB sensing.

Histone acetylation and chromatin remodeling complexes

Recent studies have demonstrated that HAT complexes act in concert with the ATP-dependent SWI/SNF and RSC (remodels the structure of chromatin)-containing chromatin remodeling complexes to facilitate DNA repair. The HAT TIP60 has been shown to acetylate core histones H2A, H3 and H4 (81,82), and cells expressing catalytically inactive TIP60 accumulate DSBs (83). Further, TIP60 and its cofactor Trapp directly bind to chromatin near DSBs and depletion of Trapp impairs DNA damage-induced H4 acetylation and impairs repair of DSBs by HR (84). Similar results were obtained with NuA4, a yeast homolog of TIP60, following HO endonuclease-induced DSBs (85). The NuA4 HAT complex binds directly to sites of DSBs and occur concomitantly with appearance of γ -H2AX (85). TIP60 forms a stable complex with ATM, and activates ATM by acetylation (86). The catalytic activity of TIP60 is stimulated in response to DNA damage, but does not appear to be regulated by ATM. As is the case with hMOF, it has been suggested that TIP60 functions upstream of ATM, possibly by sensing DNA damage-induced chromatin changes with subsequent signaling to ATM. Overall, histone acetylation appears to both unwind chromatin and create a binding platform to promote recruitment of remodeling complexes.

The INO80 complex, including the INO80 conserved member of the SWI/SNF family, has long been known to regulate transcription at RNA Pol II promoters through chromatin remodeling. More recently, it was observed that INO80 is recruited to γ -H2AX near DNA DSBs, and yeast mutants of INO80 are hypersensitive to damaging agents and HO endonuclease, providing one of the first examples of SWI/SNF ATPase participation in DNA repair (87,88). Interestingly, the actin-related protein Arp4 in yeast participates in both the NuA4 HAT complex and the INO80/SWR1 complex providing further support for the concerted action of histone modification and chromatin remodeling in the DSB response (85). The exact role of INO80 has yet to be clearly defined. Trapp-TIP60-INO80 activity may be limited to local chromatin unwinding since chromatin relaxation alone is sufficient to rescue the defects of Trapp deficiency (84). However, there is also evidence that INO80 could promote removal or sliding of histones proximal to the DSB to allow 5' to 3' strand resection and generation of a 3'-ssDNA overhang available for binding by Rad51 and homologous strand invasion (89,90). Finally, it is possible that TIP60 acetylation may create specific protein binding sites. In support of this, bromodomain proteins and some transcription factors bind preferentially to acetylated histones (91-94).

ASSEMBLY OF ADAPTOR PROTEINS ON CHROMATIN: ROLES FOR UBIQUITYLATION AND METHYLATION

DNA DSBs induce a local decrease in the density of the chromatin fiber, thus providing access for the damage sensor and adaptor proteins required for repair. MDC1 has been termed a master regulator in restructuring higher order chromatin in response to DSBs. MDC1 is one of the initial proteins to accumulate at the site of DSBs in a γ -H2AX-dependent manner (95) where it is phosphorylated by ATM. The MDC1/ γ -H2AX association is required for retention of multiple DDR proteins at the sites of damage, including NBS1, ATM, 53BP1 and BRCA1 (95–97). A positive feedback loop concentrates ATM at γ -H2AX molecules near DSBs via its interaction with MDC1, thus facilitating additional phosphorylation of adjacent H2AX molecules and amplification of the damage signal (96,97). It is not yet clear whether binding of MDC1 to γ -H2AX is sufficient to promote chromatin restructuring to increase accessibility of histone residues to adaptor proteins, or if the MDC1/ γ -H2AX complex facilitates intermediate modifications of the flanking chromatin to increase affinity for adaptor proteins. MDC1 also plays a role in regulating termination of repair as binding of MDC1 to γ -H2AX leads to shielding of the γ -H2AX C-terminal tail from dephosphorylation that would promote dissociation of chromatin repair complexes (97).

RNF8 binding provides an important link between the assembly of ‘early’ factors (NBS1, ATM and MDC1) and ‘late’ factors (53BP1, BRCA1) to γ -H2AX chromatin flanking DSBs (19,98–100). Depletion of RNF8 leads to an impaired IR-induced G2/M checkpoint and IR hypersensitivity, as well as disrupting BRCA1, 53BP1 and RAP80 IRIF (19,98,101). RNF8 contains a RING-finger domain suggestive of E3 ligase activity, and Fork-head associated (FHA) domain that recognize amino acid residues flanking a central phosphorylated residue. Two groups reported that RNF8 is responsible for IR-induced diubiquitylation of γ -H2AX and presented a model for chromatin reorganization and the sequential binding of adaptor proteins in response to DSBs (19,98). In the overall model, RNF8 is recruited to breaks by ATM phosphorylation of MDC1. Consistent with this, the RNF8 FHA domain interacts directly with a cluster of TQXF ATM phosphorylation target sites on MDC1, and MDC1 deficient cells lack IR-induced foci of RNF8 (98). RNF8 is then responsible for IR-induced H2AX ubiquitylation but not phosphorylation. Ubiquitylation likely occurs at serine 139 since H2AX-deficient MEFs reconstituted with wild-type H2AX, but not a H2AX S139A mutant, thus show IR-induced H2AX ubiquitylation (98). In contrast, reconstitution of H2AX-deficient mouse embryonic fibroblasts (MEFs) with a K119R or K120R mutant disrupts endogenous monoubiquitylation, but not IR-induced ubiquitylation (98). RNF8 interacts with the E2 ubiquitin conjugating enzyme UBC13 that has also been implicated in ubiquitylation following DNA damage (102) and DDR (98,100,103). Ubiquitylation of H2AX facilitates recruitment and retention of BRCA1 via the ubiquitin interaction motif of RAP80 and the mediator protein ABRA1 (104).

Yeast two hybrid assays also suggest that RAP80 interacts with Ubc9 (105), a SUMOylation enzyme that is required for damage tolerance and damage-induced recombination in *S. cerevisiae* (106).

Botuyan and coworkers (107) reported that lysine methylation on histone H4 recruits mammalian DNA repair factor 53BP1, and its putative fission yeast homolog Crb2, to DNA DSBs through direct binding. Using X-ray crystallography and nuclear magnetic resonance spectroscopy, they showed that 53BP1 and Crb2 contain tandem tudor domains that interact with histone H4 specifically dimethylated at lysine 20 (H4-K20me2). The structure of the 53BP1/H4-K20me2 complex revealed a unique five-residue binding cage in 53BP1 that is remarkably conserved in the structure of Crb2, and that best accommodates a dimethyllysine but excludes a trimethyllysine, thus explaining the methylation state-specific recognition of H4-K20. Thus, these studies revealed an evolutionarily conserved molecular mechanism of targeting DNA repair proteins to DSBs by direct recognition of H4-K20me2 (107). Although the related *S. cerevisiae* Rad9 also contains a tandem tudor domain, structural and charge differences prevent a similar histone interaction but instead favor DNA binding through the second tudor fold (99). Botuyan and coworkers (107) also reported that Crb2 interacts with both histones and DNA suggesting that the tudor folds enable multi-contact interactions with chromatin.

Although RNF8-mediated H2AX ubiquitylation appears required for 53BP1 recruitment, the exact mechanism remains unclear. In particular, the link between ubiquitylation and direct tudor domain binding of 53BP1 to methylated H4 is not well defined. Further, suppression of DOT1L, the enzyme that methylates lysine 79 of histone H3, inhibits recruitment of 53BP1 to DSBs (108) suggesting that this residue also acts as a target of 53BP1. It is possible that histone ubiquitylation, acetylation and methylation all contribute to a local relaxation of the DSB flanking region thus allowing for complete retention of BRCA1 and 53BP1. Alternatively, ubiquitylation and acetylation may stimulate Trapp-TIP60 HAT activity resulting in exposure of methyl residues important in 53BP1 recruitment. Further 53BP1 can function in DSB repair in XRCC4-dependent pathways of DSB repair requiring interaction with H4K20me2 but not H2AX (109).

DSB SIGNALING THROUGH THE PIKK KINASES

In response to DNA DSBs, a complex network of cell-cycle checkpoint proteins is activated, resulting in cell-cycle arrest at all three DNA damage cell-cycle checkpoints (G1-S, intra-S and G2-M). The signaling molecules that orchestrate the DDR, are the phosphatidylinositol-3 kinase-related (PIKK) class of protein kinases; ATM, ATR and DNA-PK.

ATM

The ATM protein belongs to a growing family of PIKK kinases and functions as an intrinsic part of the cell-cycle machinery that surveys genomic integrity, cell-cycle

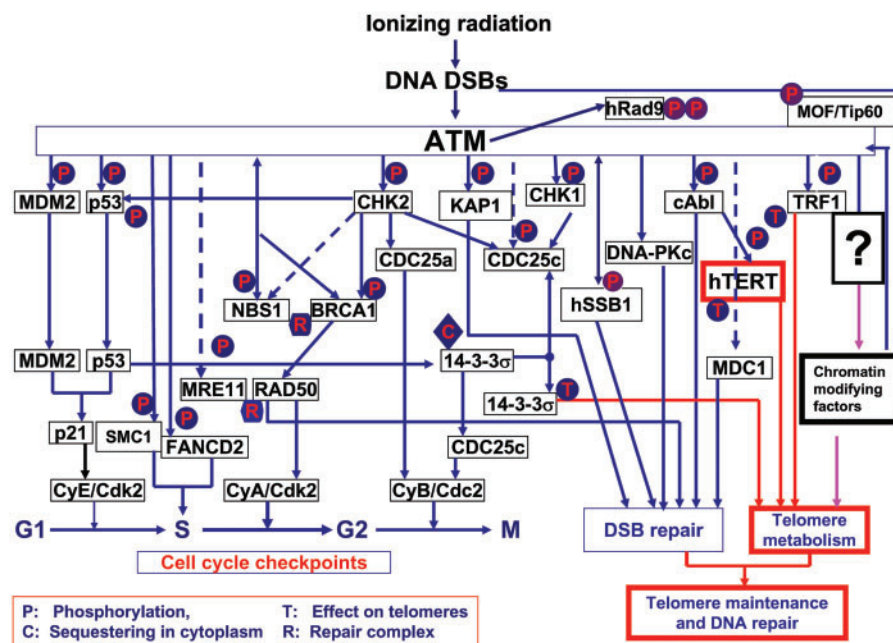


Figure 1. ATM activation in response to DNA DSBs. ATM kinase activity increases immediately after DSBs occur in DNA following exposure to IR. ATM mediates the early stages of the rapid induction of several signaling pathways, which include activation of the DNA-DSB pathway, regulation of the cell-cycle checkpoint controls, activation of stress responses and maintenance of telomeres. 'P' with solid arrows indicates reported phosphorylation events; dashed arrows represent possible signaling steps and do not imply direct interaction between proteins; 'C' indicates sequestering in cytoplasm; 'R' indicates repair complexes; and 'T' indicates a role for the protein in telomere metabolism. BRCA1, breast cancer susceptibility gene product 1; c-Abl, Abelson protein tyrosine kinase; CDK, cyclin-dependent kinase; CHK, checkpoint kinase; FANCD2, Fanconi anaemia protein; JNK, Jun N-terminal protein kinase; MRE11, meiotic recombination 11 gene product; MDM2, 'mouse double minute 2' (p53-binding protein); NBS1, Nijmegen breakage syndrome 1 protein (p95); SMC1, 'structural maintenance of chromosome' 1; RAD50, a radiation-damage-repair-associated protein; TRF1, telomere-repeat-finding factor 1; hTERT, human catalytic unit of telomerase; hMOF, the human ortholog of the *Drosophila* MOF gene (males absent on the first); hSSB1, the human ssDNA-binding protein 1; KAP1, KRAB-associated protein.

progression and processing of DNA damage (6,48). ATM protein kinase is primarily activated in response to DNA DSBs caused by IR or radiomimetic drugs, and also detects DSBs during meiosis or mitosis, or breaks consequent to damage by free radicals (Figure 1) (110). It shows similarity to several yeast and mammalian proteins involved in meiotic recombination and cell-cycle progression, namely, the products of *MEC1* in the budding yeast *S. cerevisiae* and *RAD3* in the fission yeast *S. pombe* (111,112) and the Tor proteins found in yeasts and mammals (113,114). Cells deficient in ATM have been shown to have a high frequency of spontaneous chromosomal aberrations, high rates of intrachromosomal recombination and error-prone recombination (6,48). Such cells have higher initial and residual chromosomal aberrations in the G1- and G2-phases after IR exposure as determined by premature chromosome condensation (1,2,72). Cells defective in ATM function also display higher frequencies of chromosomal aberrations after IR exposure (72). Mice mutated in the *Atm* gene display similar pleiotropic defects (115–117).

ATM is observed at the sites of DNA damage, where it is autophosphorylated (73,118) and is dissociated from its nonactive dimeric form into the active monomeric form (73,118). Lee and Paul (20) reported that ATM stimulation appeared to be primarily through an increase in substrate recruitment by ATM. From these findings, we proposed that the Mre11 complex binds to ATM,

inducing conformational changes that facilitate an increase in the affinity of ATM toward its substrates.

Detection and signaling of DNA damage are mediated through downstream targets of ATM (Figure 1) (48). ATM is a 'hierarchical kinase', capable of initiating multiple pathways simultaneously (48). After recruitment to sites of DNA damage, ATM directly phosphorylates numerous substrates including p53, Chk1 and Chk2 which in turn target other proteins to induce cell-cycle arrest and facilitate DNA repair (Figure 2). Although ATM is known to be a central transducer of DNA damage signals, studies have also demonstrated that ATM stabilizes chromosomal V(D)J recombination DSB intermediates, facilitates DNA-end joining and prevents broken ends from participating in chromosomal deletions, inversions and translocations (4,5). Furthermore, it has been reported that ATM-mediated checkpoints block the persistence and transmission of un-repaired DSBs in developing lymphocytes (119). We reported that ATM phosphorylates ssDNA-binding proteins (SSBs) hSSB1 in response to DNA DSBs (120). This phosphorylation event is required for DNA damage-induced stabilization of hSSB1. Upon induction of DNA damage, hSSB1 accumulates in the nucleus and co-localizes with other known repair proteins. In contrast to RPA, hSSB1 does not localize to replication foci in S-phase cells and hSSB1 deficiency does not influence S-phase progression. Depletion of hSSB1 abrogates the cellular response to DSBs,

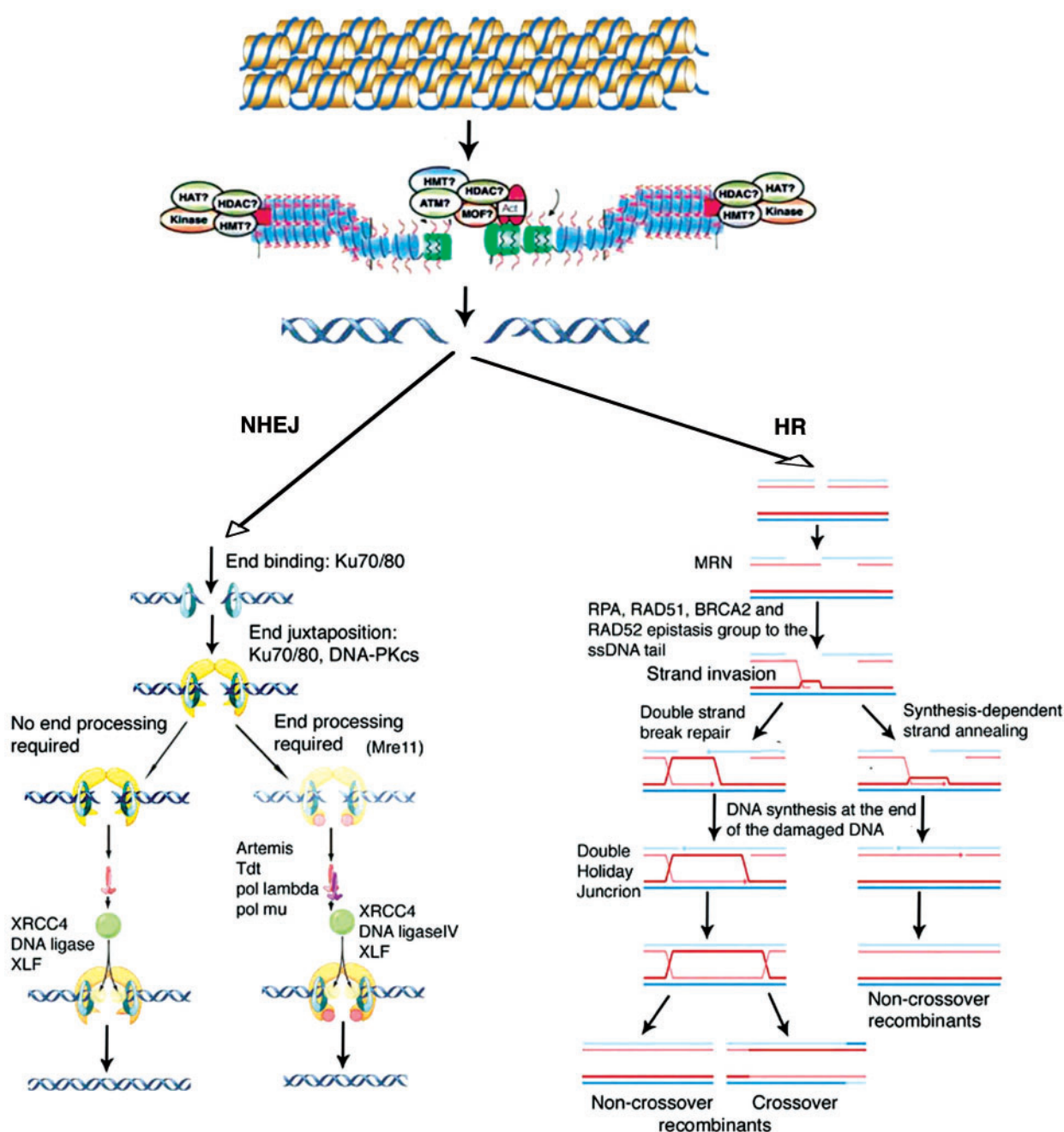


Figure 2. Major regulatory steps in DSB repair. DNA damage repair is accompanied by recognition of damage, modification of chromatin at the site of DNA damage, recruitment of repair factors and cell-cycle checkpoints. Multiple proteins with different activity for posttranslational modifications of histones present at the DNA DSB allow to open the chromatin in order to make the DNA accessible to DNA repair machinery. Histone modifications are necessary to remodel the nucleosomes during the repair process. Several proteins have been reported to have multiple functions that are involved in the regulation of the DNA DSB repair, whether the damage requires to be repaired by NHEJ or HR. A two-ended DNA DSB induced by IR are substrates for binding of the Ku70/Ku80 heterodimer. Ku70/Ku80 bound to DNA ends recruits DNA-PKcs to the ends and promotes their juxtaposition. If no further processing of the ends is required, the additional core components of nonhomologous DNA end-joining, XRCC4, DNA ligase IV and XLF (XRCC4-like factor also known as Cernunnos) can promote the rejoining reaction. If the two-ended DNA DSB require end processing, then such processing may require the activities of the nuclease Artemis and/or the DNA polymerase Tdt, pol lambda and pol mu. The Ku heterodimer likely plays a central role in orchestrating the activities of the proteins involved in NHEJ. The exact nature of the active complex is currently undefined, but the transient reversible interaction of the processing factors with the core components provides great flexibility in the combination of broken ends that can be rejoined because the process does not require strict order in which the processing factors engage or in which the four strands will be processed. In general, the final stage of NHEJ is the ligation of DNA ends catalyzed by XRCC4-ligase IV, and this process is promoted by Cernunnos-XLF in an unknown way. DNA DSBs repaired by HR involve various steps. For the promotion of invasion, several proteins like Rad51, Dmcl are loaded on the replicated DNA of the intact homolog by a single-strand tail of the resected DSB. The close pair of parallel lines represent the two strands of duplex DNA. The left-hand side of the top most strand has 3' polarity. One of the two sister chromatids has damage-induced DSB. Processing results in single-stranded tails at the break with 3'-hydroxyl ends. The tails are substrates for nucleoprotein filament formation, which are directed for homology recognition and DNA strand exchange lead to joint molecule formation between the broken DNA and the intact sister chromatid.

including activation of ATM and phosphorylation of ATM targets after IR.

DNA-damage checkpoint pathways in *S. cerevisiae* are governed by the ATM homolog Tel1 and the Mre11 complex (121). In mitotic cells, the Tel1–Mre11 complex pathway triggers Rad53 activation and its interaction with Rad9, whereas in meiosis it activates Rad9 and the Rad53 paralog Mre4/Mek1. Activation of the Tel1–Mre11 complex checkpoint appears to depend upon the Mre11 complex as a damage sensor and, at least in meiotic cells, to depend on unprocessed DNA DSBs. The DSB repair functions of the Mre11 complex are enhanced by the pathway, suggesting that the complex both initiates and is regulated by the Tel1-dependent DSB signal. These findings suggest that the Mre11 complex has a role in the meiotic recombination as well (121).

ATR

While ATM is involved in damage-induced DSB response, the ATR (ATM- and Rad3-related) protein kinase primarily responds to replication stress and other forms of DNA damage, such as UV. ATR is recruited by ATR-interacting protein (ATR-IP) to the sites of DNA damage, in particular to RPA-coated ssDNA that accumulates at stalled DNA replication forks or is generated by the processing of the initial DNA damage. Once at the break, like ATM, ATR phosphorylates Chk1 and Chk2, thus inducing cell-cycle arrest.

It was previously thought that ATM and ATR had overlapping but distinct roles in the response to DNA damage. However, Trenz and coworkers (122) demonstrated that both ATM and ATR work in a coordinated manner by promoting Mre11-dependent restart of collapsed replication forks and prevent accumulation of DNA DSBs. One study showed that ATR is activated rapidly by IR and both ATM and Mre11 enhance ATR signaling (123). These investigators postulate that ATM and Mre11 may stimulate the ATR signaling pathway by converting DNA damage generated by IR into structures that recruit and activate ATR.

ATM and ATR have been regarded as important components in the machinery monitoring progression of meiotic recombination, DSB repair and homolog pairing (124), in agreement with the location of murine *Atm* throughout meiotic chromatin (125,126). Meiotic chromosomes from *Atm*-deficient mice show aberrant synapsis with unpaired axial cores, nonhomologous synapsis and fragmented SCs (44,45). Consistent with a role for *Atm* in meiosis, individuals with ataxia-telangiectasia display gonadal atrophy and spermatogenic failure, a phenotype mirrored by *Atm*-deficient mice (48). Furthermore, the yeast ATR ortholog *Mec1* is known to exert checkpoint function in the mitotic and meiotic cell cycle, and its absence mediates a defect in synapsis (112,127). *Mec1* is required for phosphorylation of replication protein A (RPA) in response to IR-induced DNA damage (128), and in turn RPA has been shown to interact with Rad51, which plays an important role in mitotic and meiotic recombination (129,130) and localizes to meiotic recombination complexes (131–133).

DNA-dependent protein kinase

The DNA-dependent protein kinase (DNA-PK) is a multi-component complex consisting of the DNA-PK catalytic subunit (DNA-PKcs) and the Ku heterodimer (Ku80 and Ku70). The DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is critical for DNA repair via the nonhomologous end joining pathway and is mutated in SCID mice. DNA-PKcs also plays a role in the signaling response. DNA-PKcs is able to phosphorylate several targets of ATM, although redundancy in function is not absolute. In ATM null or ATM kinase-dead cells, DNA-PKcs can phosphorylate H2AX, but this phosphorylation is not observed in the presence of wild-type ATM (134). In addition, histone H1 phosphorylation by DNA-PK promotes efficient DNA repair (135).

Data support a role for DNA-PK in apoptotic suppression either through its role in DNA repair or damage response signaling. A DNA-PKcs null mutation in mice exhibit blocked V(D)J coding joining but not recombinational signal (RS)-end joining or growth retardation. In SCID mice the spermatogonia are radiosensitive (136). Spermatogonial apoptosis occurs faster in irradiated DNA-PKcs-deficient SCID testis compared with their wild-type counterparts. However, p53 induction is unaffected in SCID cells (136). Similarly, intestinal crypt cells from p53 nullizygous mice are resistant to radiation-induced apoptosis, whereas apoptosis in DNA-PK(cs)/p53 double-null mice is equivalent to that seen in wild-type mice (137). These studies suggest a p53-independent apoptotic response to DNA damage utilized in the absence of DNA-PK. It should be noted, however, that mice null for DNA-PKcs do not exhibit the overall growth retardation that SCID mice do, and although null fibroblasts are radiosensitive, null embryonic stem (ES) cells are not (138).

Spontaneous apoptosis of spermatocytes occurred in the SCID testis suggesting that DNA-PKcs functions independently of the Ku proteins to promote DNA repair in these cells. The majority of these apoptotic spermatocytes are found at stage IV of the seminiferous epithelium where a meiotic checkpoint has been suggested to exist. DSBs are less accurately repaired in SCID spermatocytes that then fail to pass the meiotic checkpoint. Thus, the role for DNA-PKcs during the meiotic prophase differs from that in mitotic cells since it is not influenced by IR and is independent of the Ku heterodimer.

RECOGNITION OF DSBs IN NONDAMAGED CELLS

In addition to the random introduction of DSBs that occur as a result of cellular exposure to DNA-damaging agents, DNA DSBs are also formed in a programmed manner during development. They are generated to initiate recombination between homologous chromosomes during meiosis (3) and occur as intermediates during developmentally regulated rearrangements, such as V(D)J recombination and Ig class-switch recombination.

Initiation of meiotic recombination by DSBs

At the beginning of meiosis, each chromosome must recognize its homolog, then the two become intimately aligned along their entire lengths forming the SC, which allows for the exchange of DNA strands between homologous sequences. Meiotic recombination is initiated by DNA DSBs in a variety of organisms. Numerous studies have identified both the genomic loci of the initiating DSBs and the proteins involved in their formation.

Meiotic recombination initiates with DSBs formed by Spo11, a topoisomerase II-like protein (Spo11 in *S. cerevisiae* and vertebrates, Rec12 in *S. pombe*, Mei-W68 in *Drosophila*) (139–144). DSB formation also requires the products of at least nine other genes that act by stabilization or recruitment mechanisms. These include meiotic-specific Mei4 (145), Mer2 (146), Rec102, Rec104, Rec114 (Rec7 in *S. pombe*) (147,148), Rec103 (Ski8; Rec14 in *S. pombe*) (149) as well as the Mre11 complex (150–152). Sae2/Com1 (CtIP in mammals), discussed above, is required for DSB processing. The localized Spo11-induced DSBs appear before the formation of joint molecules, and their frequency correlates with the frequency of gene conversion and crossing-over. Meiotic DSBs also initiate DSB response checkpoints that ensure the completion of recombination before the exit from pachytene.

As expected, the lack of DSBs in yeast *SPO11*^{-/-} mutants blocks recombination initiation, synapsis and sporulation. Similarly, although viable, *SPO11*^{-/-} mice are infertile and display multiple meiotic pairing, synapsis and recombination defects (153,154). Mammalian *SPO11*^{-/-} spermatocytes and oocytes undergo elevated levels of apoptosis. Consistent with the interdependence of meiotic recombination and synapsis, DSBs may serve as the initial regulatory or structural signal required for progression through meiosis without which apoptosis occurs (60). Conversely, it is possible that the lack of DSBs in *SPO11*^{-/-} mice induces cell arrest and produces a pro-apoptotic signal (153). Spo11 could also serve a structural role secondary to DSB formation (154) leading to the prediction that separation of functional mutants will demonstrate independent catalytic and structural roles as well as an understanding of apoptotic signaling in the absence of Spo11 function. Although highest in testis and ovary, the expression of mammalian Spo11 and alternative transcripts are also detected in several somatic tissues including lymphocytes (153,154), suggesting a possible role of these gene products in other DSB-mediated developmental programs, such as somatic hypermutation or class switching (155,156). However, analysis of *SPO11*^{-/-} mice to date has not revealed any role of Spo11 outside of meiosis (157).

In mammals, many of the *RAD52* epistasis group (Figure 2) homologs are expressed in multiple tissue types, but with higher levels of expression seen in the testis and proliferating cells, indicating their involvement in both meiotic and mitotic recombination. The protein–protein interactions among various members of the *RAD52* epistasis group suggest that two different

complexes are involved in DSB-induced recombination; the first is involved in presynaptic functions including the processing of the DSB ends, and the second involved is in synaptic functions for invasion, creation of repair intermediates and resolution (158).

V(D)J recombination

During early B- and T-cell development, the exons that encode Ig and T-cell receptor (TCR) variable regions are assembled from germline variable (V), diversity (D) and joining (J) segments via V(D)J recombination. The reaction is initiated when the RAG1 and RAG2 protein complex introduces DNA DSBs at specific sites within the border of two gene segments and their flanking recombination signals. RAG2 serves not only an enzymatic role in the reaction but also a chromatin stabilizing role. RAG2 (aa 414–487) contains a noncanonical plant homeodomain (PHD) finger that specifically binds trimethylated lysine 4 of histone H3 (H3K4me3). Abrogation of this interaction by either mutation of RAG2 or reduction of H3K4me3 levels impairs V(D)J recombination *in vivo*. Importantly a set of patients with the immunodeficiency Omenn's syndrome are mutant for a conserved tryptophan (W453) in this region (159,160). Although it was known that H3K4me3 functions in modulating transcription, this is the first demonstration for a H3K4me3 role in recombination and suggests that this type of linkage may exist in other DNA repair pathways as well. A recent study by Bredemeyer and coworkers (4) elegantly demonstrated that ATM functions directly in the repair of Rag-induced chromosomal DNA DSBs by maintaining DNA ends in their repair complexes generated during lymphocyte antigen receptor gene assembly. This study explains the increase in lymphoid tumors that carry translocations involving antigen receptor loci associated with ataxia-telangiectasia.

HUMAN DNA DSB DISORDERS

Within the past decade, a large amount of new information has contributed to the understanding of DNA DSBs sensing and processing and its relationship to human diseases (13). Such studies profoundly altered the conceptual model of DNA DSB repair. Until 1995, the majority of information about the induction and repair of DNA DSBs came from budding and fission yeast (161). Since then, the focus has shifted to the study of a number of human genetic disorders that are characterized by a defective DSB responses (162) (Table 2). These disorders exhibit a number of common characteristics such as developmental defects, immunodeficiency, neurological degeneration and cancer predisposition. The use of cell lines and mouse models of these DSB-disorders has greatly enhanced our understanding of the cellular processes that regulate the maintenance and processing of DSBs.

Mutations in Mre11 and Nbs1 of the Mre11 complex result in ataxia-telangiectasia-like disorder (ATLD) and Nijmegen breakage syndrome (NBS), respectively. As yet, no human condition has been identified for mutated Rad50. Knockout mouse models of all three genes are

Table 2. DNA DSB disorders

Syndrome	Gene	Phenotype
AT	<i>ATM</i>	Immunodeficiency, neurodegeneration, cancer predisposition
ATLD	<i>Mre11</i>	Milder form of AT, no reported cancer phenotype
NBS	<i>Nbs1</i>	Immunodeficiency, developmental defects, cancer predisposition
Seckel syndrome	<i>ATR</i>	Developmental defects, no reported cancer phenotype
Fanconi anaemia	<i>BRCA2 (FANCD1)</i>	Immunodeficiency, developmental defects, cancer predisposition
RS-SCID	<i>Artemis</i>	Immunodeficiency, no reported cancer phenotype
LIG4 syndrome	<i>LIG4</i>	Immunodeficiency, developmental defects, cancer predisposition

embryonic lethal, highlighting the significance of this complex in normal cellular functions (42,163,164). Mutations in proteins which belong to the PIKK protein family, ATM and ATR, result in ataxia-telangiectasia and Seckel syndrome (Table 2). In addition, there are several syndromes associated with proteins that are involved directly in the DSB repair process. Defective LIG4 and Artemis result in LIG4 syndrome and severe combined immunodeficiency (SCID), respectively. Both proteins function in the NHEJ pathway. Specific BRCA2 mutations give rise to Fanconi anemia (complementation group FANCD1) (Table 2), which results in defective HR. The broad spectrum of clinical features of these DSB disorders clearly highlights the importance in regulating this type of DNA lesion.

FUTURE STUDIES AND DIRECTIONS

Considerable advances have been made in recent years in elucidating the mechanisms and pathways by which cells regulate the repair of DSBs. However, what can we expect in the coming decade about the repair of DNA DSBs?

It is becoming increasingly evident that the higher order nuclear organization of chromatin (Figure 2) plays a key role in a cell's ability to initiate signaling cascades in response to DSBs. Certainly, we will learn more about the function of chromatin modifying factors including the interdependence of chromatin modification and DDR signaling pathways, and their enzymatic activities and mechanisms of regulation for DNA DSB repair by both NHEJ and HR. Further, we know little about the interdependence between chromatin remodeling, signaling and DDR in euchromatin versus heterochromatin and transcribed versus untranscribed regions.

Although individual repair HR and NHEJ pathways have been well defined, it still remains unclear that the mechanism by which cells regulate DNA repair process, will be utilized to repair a specific DSB. Further investigation is needed to determine if specific types of damage, i.e. IR, UV or radiomimetic drug-induced DSBs, trigger either NHEJ or HR pathways. It is clear that during certain phases (G1) of the cell cycle that NHEJ is the predominant mode of repair. However, during late S and G2 phases both HR and NHEJ can contribute to DSB repair. It is interesting to speculate that the nature of the broken ends of the DNA and their initial end processing may ultimately determine whether the DSB is repaired by HR or NHEJ. Recent studies demonstrated that CtIP and the

related yeast Ctp1 are recruited to DSBs exclusively in S and G2 phases (29,31). Furthermore, transcription of *ctp1* is periodic with expression coinciding with the start of DNA replication (31). A role for DNA-PK in both H1 phosphorylation and efficient NHEJ suggests that specific chromatin alterations may create structures that favor recruitment or retention of a specific subset of DNA repair proteins and thus favor a particular DNA repair pathways. In addition, recruitment of the RSC chromatin remodeling complex to promote removal of histones from chromatin at HO-induced breaks is dependent on MRE11 and Ku70, and mutants in two components of RSC, Rsc8 and Rsc30, impair NHEJ but not HR or damage-induced checkpoint activation (165,166). Further support for this idea comes from the link between INO80 activity and removal of histone proximal to DSBs to allow for resection and generation of ssDNA tails for Rad51-mediated initiation of HR (89,90). These are some of the first data to emerge that provide insight into the cell-cycle control of HR and NHEJ, and more data in this area are sure to emerge in the next few years.

We currently have a reasonably good understanding of how cells activate the DDR. However, little is known about how cells downregulate the DSB response and restore normal chromatin structure once damage is repaired. It is becoming increasingly evident that just as proteins are activated by phosphorylation and acetylation in response to the DSBs, they are also subjected to other modifications to return them to a ready but inactive state. The Pph3 phosphatase in yeast and PP2A in mammalian cells have been shown to directly dephosphorylate γ -H2AX but whether dephosphorylation is of chromatin-associated γ -H2AX or after displacement or turnover occurs is unclear (167,168). Histone deacetylases are also associated with sites of DSBs and may promote efficient repair of DSBs by HR (169). These results raise the question of the balance between removal of histone modifications in chromatin and exchange of modified histones for unmodified ones. Just as the DDR entails a complex coordination of signaling, adaptor, and effector proteins along with chromatin modifications, there is surely an equally complex and coordinated sequence of events regulating the restoration of chromatin structure and cell-cycle progression following repair.

Greater insight into the cellular control of DNA DSBs will be important not only for increased understanding of cellular responses to stress in general, but also for the etiology of some cancers and development of new

therapeutic treatments for individuals with dysfunctional DSB repair pathways. Proteins involved in chromatin structural alterations to facilitate DNA repair may be altered in disease and some cancers. It is now appreciated that chromatin modifying proteins play a role in DDR, but some also interact with multiple known oncogenes. Trapp interacts with c-Myc, E2F and E1A, and Trapp antisense RNA blocks c-Myc- and E1A-mediated oncogenic transformation. As with other areas of basic research, the understanding of the significance of these interactions to tumor development will be of increasing interest in the coming years and may enable the design and application of novel therapies.

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